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Oxidation and Reduction of Sulfite by Chloroplasts and Formation of Sulfite Addition Compounds[1]

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ABSTRACT

After exposing intact chloroplasts isolated from spinach (*Spinacia oleracea* L. cv Yates) and capable of photoreducing CO_2 at high rates to different concentrations of radioactive sulfite in the light or in the dark, $^{35}SO_2$ and $H_2{}^{35}S$ were removed from the acidified suspensions in a stream of nitrogen. Remaining activity could be fractionated into sulfate, organic sulfides, and sulfite addition compounds. When chloroplast suspensions contained catalase, superoxide dismutase and *O*-acetylserine, the oxidation of sulfite to sulfate was slower in the light than the reductive formation of sulfides that exhibited a maximum rate of about 2 micromoles per milligram chlorophyll per hour, equivalent to about 1% of maximum carbon assimilation. Both the oxidative and the reductive detoxification of sulfite were very slow in the dark. Oxidation was somewhat, but not much, accelerated in the light in the absence of *O*-acetylserine, which caused a dramatic decrease in the formation of organic sulfides and an equally dramatic increase in the concentration of sulfite addition compounds whose formation was light-dependent. The sulfite addition compounds were not identified. Addition compounds did not accumulate in the dark. In the light, the electron transport inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea, diuron, decreased not only the reduction, but also the oxidation of sulfite and the formation of addition compounds.

In those parts of Europe where brown coal is burnt, SO_2 is a major air pollutant. At high atmospheric concentrations, SO_2 causes necrosis in leaves and finally death of plants. Lower concentrations reduce growth (17). The resistance of plants to SO_2 is reduced on soils that have been depleted of nutrients after prolonged exposure to acid rain (26).

SO_2 enters leaves or needles mainly through the stomata. It is highly soluble in aqueous phases, but can also penetrate biomembranes easily (21). Hydration and dissociation of the hydration product sulfurous acid produces protons, HSO_3^-, and SO_3^{2-}. In the cytoplasm of mesophyll cells, sulfite[3] is taken up by the chloroplasts via the phosphate translocator (11).

Chloroplasts are sites of both detoxification and toxic effects of SO_2. Sulfite can change the activity of enzymes, disturb membranes by radical reactions, or inhibit the thylakoid membrane electron transport chain (5, 23, 28).

However, chloroplasts of mesophyll cells also are able to metabolize sulfite in light-dependent reactions. Two pathways are possible (25). One possibility is the oxidation of sulfite to sulfate. It can proceed via a radical chain reaction (3). Radicals may cause damage by their reaction with biomolecules. Sulfate is actively transported into the vacuoles (16), but can also be reduced slowly in the so-called "bound pathway" of cysteine synthesis (1). The other pathway is the direct reduction of sulfite (13). The product is the highly toxic intermediate hydrogensulfide, which is metabolized to the amino acid cysteine, although a small part of it is released into the atmosphere (6, 10).

Different plants exhibit different sensitivity to SO_2. Apparently, they are differently equipped to deal with this air pollutant. Little is known of the SO_2 detoxification capacity of intact chloroplasts. In previous work, it has been demonstrated that in the presence of *O*-acetylserine, a cosubstrate for cysteine biosynthesis, and in the presence of an effective scavenging system for oxygen radicals, spinach chloroplasts may exhibit net O_2 evolution in the light in the presence of sulfite (9). This shows that under appropriate conditions reduction may successfully compete with oxidation for available sulfite. The present investigation presents kinetic data on the ability of spinach chloroplasts to either oxidize or reduce sulfite. It will also be shown that under conditions in which both reactions are slow, sulfite addition compounds accumulate in the light.

MATERIALS AND METHODS

Plant Material

Chloroplasts were isolated from 8- to 12-d-old leaves of spinach (*Spinacia oleracea* L. cv Yates), which was grown in a green house in earth culture. The light period was 11 h/d. The temperature was about 10°C at night and between 18° and 30°C during the day.

Isolation of Intact Chloroplasts

Intact chloroplasts were isolated by a modification of the method of Jensen and Bassham (15). The isolation medium

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[2] Died: June 10, 1991.

[3] In solution, SO_2 is in equilibrium with dissolved sulfur dioxide ($SO_2 \cdot H_2O$), bisulfite (HSO_3^-), and sulfite (SO_3^{2-}). The latter is the predominating molecular species at cytoplasmic pH values. Concentration data given in this paper for sulfite include also the other molecular species.

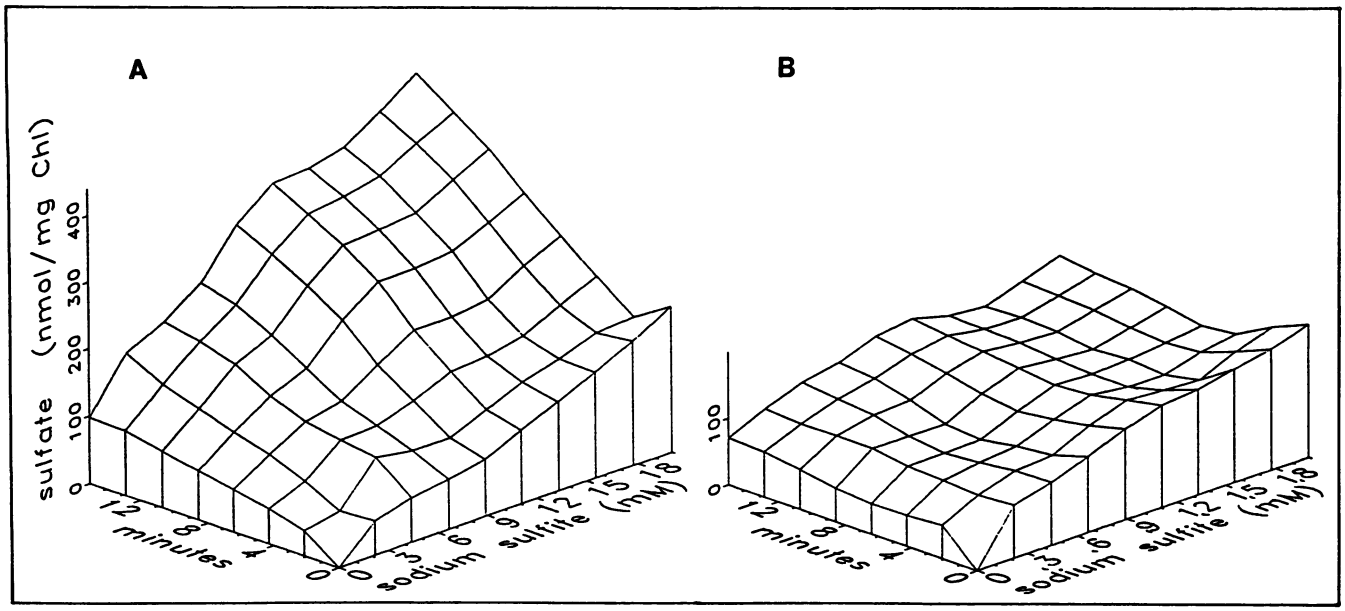

Figure 1. Oxidation of sulfite to sulfate in isolated spinach chloroplasts in the presence of 10 mM OAS in the light (A) and in the dark (B).

A contained 300 mM sorbitol, 50 mM Mes-buffer, 30 mM KCl, 2 mM EDTA, 2 mM $MgCl_2$, and 0.25 mM KH_2PO_4. The pH was adjusted to 6.1 with KOH; 3 mM cysteine and 1.25 mM Na_2-ascorbate were added before grinding the leaves in a blender. The homogenate was filtered through a nylon net (20 μm). Chloroplasts were isolated in two centrifugation steps. The first centrifugation was at 2700*g* for 60 s, and the second at 2200*g* for 45 s. Between the centrifugations, the sediments were taken up in medium A. After the last centrifugation, the chloroplasts were suspended in medium C. Medium C differed from medium A in that 50 mM Hepes buffer was used instead of Mes buffer, and pH was 7.6. The percentage of chloroplasts that had retained their envelopes was about 80% as determined by the method of Heber and Santarius (12). Chl was determined according to the method of Arnon (2). Under saturating illumination, the rate of CO_2-dependent O_2 evolution by the chloroplasts was between 100 and 200 μmol (mg Chl·h)$^{-1}$.

Experimental Conditions

Chloroplasts suspended in medium C were stirred in a glass cuvette at 20°C. The Chl concentration was 100 $\mu g \cdot mL^{-1}$. Illumination was provided by a slide projector. A red cutoff filter (RG 610 from Schott, Mainz, Germany) was used to remove light below 600 nm. The quantum flux density was 2500 $\mu E \cdot m^{-2} \cdot s^{-1}$; 100 units·mL^{-1} SOD[4] (Sigma, Deisenhofen, Germany) and 2500 units·mL^{-1} catalase (Boehringer Mannheim, Germany) were added to scavenge O_2 radicals and to destroy H_2O_2 produced by contaminating envelope-free chloroplasts; 10 mM *O*-acetylserine was also added where indicated. After 5 min in the dark, the light was switched on, and after 30 s in the light, radioactively labeled sodium sulfite was added at different concentrations.

Determination of Radioactive Products

Samples were removed from the reaction mixtures at different times. Aliquots were measured directly in a liquid scintillation counter (Kontron, Eching, Germany). There were no differences in radioactivity between the first and the last sample taken, showing that there was no loss of volatile sulfur components during the experiment. Another series of aliquots (150 μL) was given to equal volumes of 2.5 N HCl. SO_2 and other volatile compounds such as H_2S were flushed out by a stream of N_2 (15 min). Subsequently, the samples were taken to dryness. The residue was dissolved in 200 μL sodium succinate buffer (260 mM, pH 4.5). Radioactivity measured in 50-μL samples showed the amount of the non-volatile reaction products such as sulfate, organic sulfides, and sulfite addition compounds; 25 μL of 8 mM sodium sulfate was added to the remaining 150 μL, followed by 25 μL of 800 mM $BaCl_2$. After 1 h at room temperature and 1 h on ice, $BaSO_4$ was removed by centrifugation; 50-μL aliquots were taken from the supernatant, and the remaining radioactivity was measured. It revealed the concentration of organic sulfides and of undefined addition compounds; 11 μL of 0.5 M DTT were then added to 100 μL of the supernatant, and the mixture was incubated at 60°C for 6 h. DTT served as a reductant to liberate sulfite from addition compounds. After incubation, the samples were left to stand at room temperature for 30 min; 14 μL of 8 mM sodium sulfate were added to aid precipitation of radioactive sulfate and sulfite as insoluble barium salts. The barium salts were again removed by centrifugation. Radioactivity was measured in 80 μL of the supernatant. It indicated the content of organic sulfides. Thus, radioactivity measurements in four fractions permitted, by

[4] Abbreviations: SOD, superoxide dismutase (EC 1.15.1.1.); OAS, *O*-acetyl-L-serine.

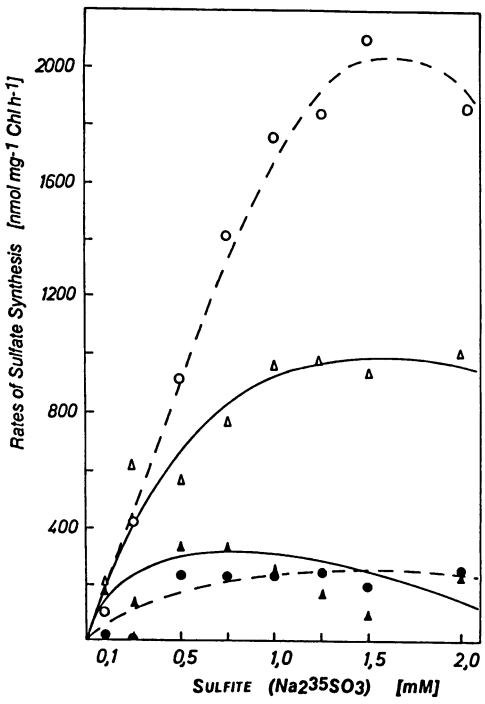

Figure 2. Rates of oxidation of sulfite to sulfate in isolated spinach chloroplasts. △——△; In the presence of OAS in the light; ▲——▲, in the presence of OAS in the dark; O- - -O, without OAS in the light; ●- - -●, without OAS in the dark.

subtraction, the calculation of the content of three groups of sulfur compounds, which are formed from sulfite in darkened or illuminated suspensions of intact chloroplasts. They were: sulfate, organic sulfides, and sulfite addition compounds, which were not further characterized.

RESULTS AND DISCUSSION

Oxidation of Sulfite to Sulfate

Isolated washed thylakoids oxidize added sulfite under high-intensity illumination in a chain reaction at rates that may be far higher than maximum rates of light-dependent electron transport if no scavengers for activated O_2 are added (3). Oxidation is initiated by O_2 radicals, which are formed in the Mehler reaction. However, when suspended in a medium that contained catalase and SOD, isolated intact chloroplasts oxidized sulfite only slowly in the light (Fig. 1A). The added enzymes scavenge O_2 radicals, which are formed or appear outside the intact chloroplasts and destroy the H_2O_2 formed. Sorbitol, which served as osmoticum, also suppresses radical reactions (3). In the dark, oxidation of sulfite was very slow (Fig. 1B). Radioactive sulfate found in the suspension immediately after sulfite was added originated only in part from sulfate contaminating the radioactive sulfite preparation. It appeared that oxidants present in the chloroplast suspensions caused some rapid initial oxidation. The subsequent increase in sulfate levels was very slow and dependent on the concentration of sulfite.

In the experiment of Figure 1, 10 mM *O*-acetylserine was present to enable the reductive formation of cysteine from

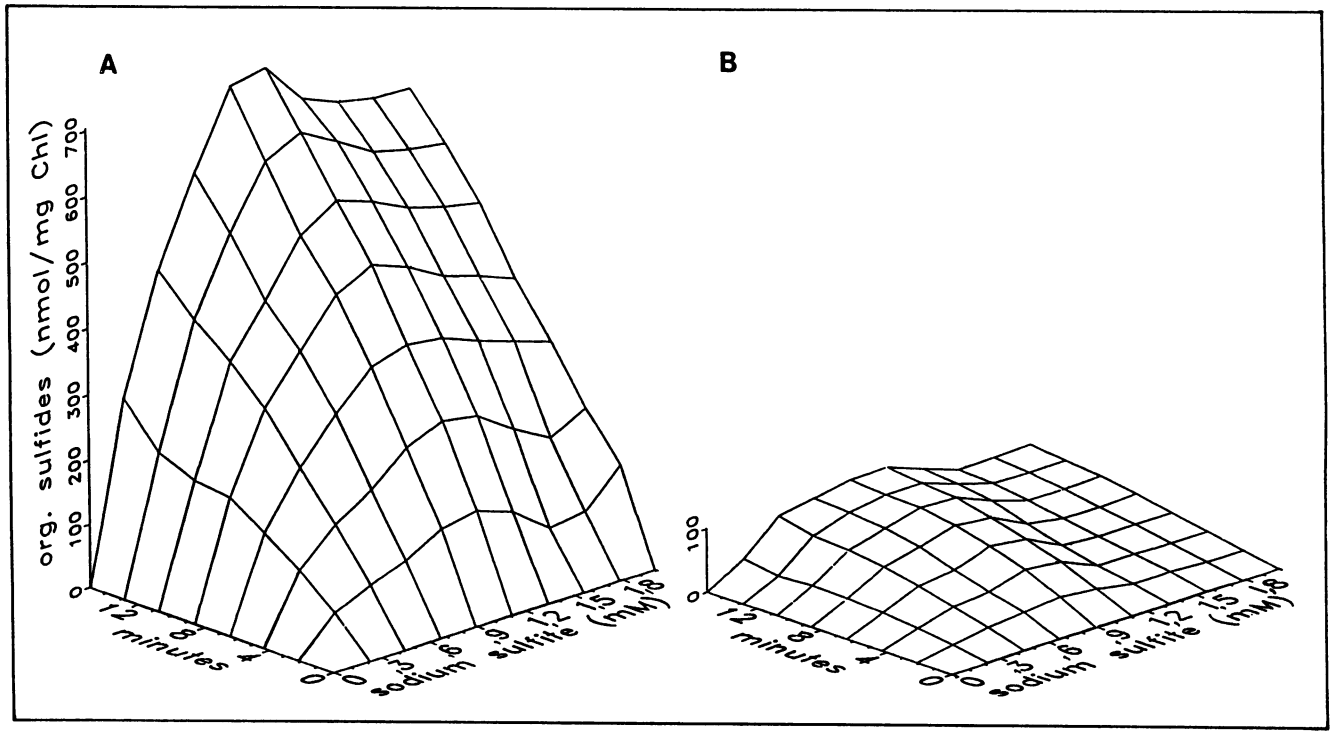

Figure 3. Formation of organic sulfides in the light by a suspension of isolated spinach chloroplasts in the presence of 10 mM OAS (A) and in its absence (B).

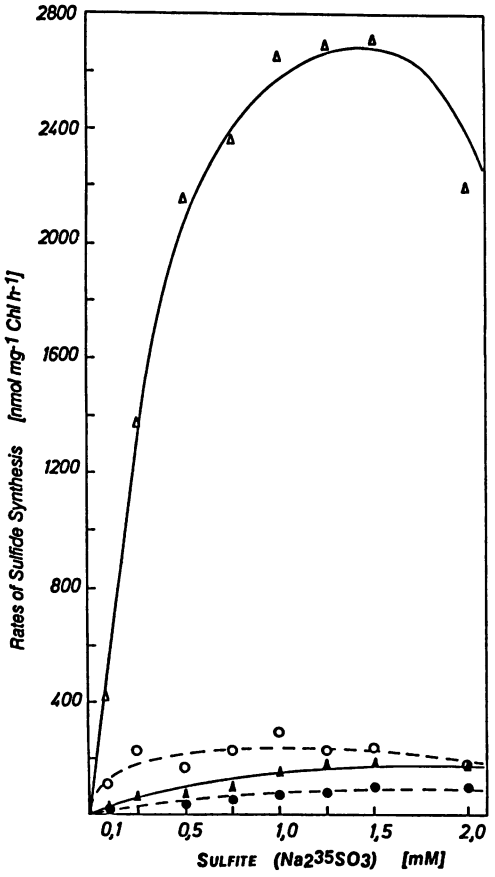

Figure 4. Rates of reduction of sulfite to organic sulfides in isolated spinach chloroplasts. △——△, In the presence of OAS in the light; ▲——▲, in the presence of OAS in the dark; O– – –O, without OAS in the light; ●– – –●, without OAS in the dark.

sulfite. When it was absent, the slow oxidation seen after the initial fast phase was accelerated at higher sulfite concentrations. Figure 2 shows sulfite oxidation rates. They were evaluated by plotting the increase of the sulfate concentration between 8 and 15 min after the addition of sulfite against the concentration of added sulfite. In this time range, the sulfate concentration increased continuously in all experiments.

In the presence of *O*-acetylserine, oxidation followed Michaelis-Menten kinetics up to a concentration of 1 mM sulfite. The apparent Michaelis constant was 0.5 mM SO_3^{2-}. In the absence of OAS, sulfate formation was increased at elevated sulfite levels compared to oxidation in the presence of OAS. Maximum light-dependent sulfite oxidation usually did not exceed 2 μmol (mg Chl·h)$^{-1}$. This corresponds to only about 1 to 2% of commonly observed rates of CO_2-dependent O_2 evolution. Slow oxidation by intact chloroplasts, which contrasts sharply to the fast oxidation by washed thylakoids, shows that intact chloroplasts are excellently equipped to scavenge O_2 radicals even when sulfite concentrations are high. To effectively decrease light-dependent sulfate formation in preparations of intact chloroplasts, it is sufficient to suppress radical formation by contaminating thylakoids in the suspending medium by SOD and catalase (9). Additional protection can be provided *in vitro,* and is provided *in vivo,* by the antioxidant ascorbate.

Light-dependent oxidation was largely but, even at the very high concentration of 50 μM, not completely suppressed by the herbicide DCMU that inhibits the production of O_2 radicals at the reducing side of PSI by inhibiting linear electron flow (data not shown).

In the dark, sulfite oxidation was slow, below 300 nmol SO_3^{2-} (mg Chl·h)$^{-1}$, and largely independent of the presence of OAS.

Reduction of Sulfite

Another pathway of sulfite metabolization is found in the direct reduction by sulfite reductase. This enzyme reduces sulfite to sulfide in a ferredoxin-dependent reaction (13). It was found to be active in spinach chloroplasts (18). Formation of organic sulfides by intact chloroplasts in the light is shown as a function of the concentration of sulfite in the presence of OAS in Figure 3A and without added OAS in Figure 3B. When OAS was absent, little organic sulfide was formed in the light. In its presence, however, synthesis of organic sulfide from sulfite was actually faster in the light than oxidation of sulfite to sulfate (Fig. 1). Reduction of sulfate is far slower than reduction of sulfite (9). Obviously, sulfate is not an intermediate in observed sulfite reduction.

Rates of formation of organic sulfides (Fig. 4) were comparable to rates of the light-dependent synthesis of cysteine (9), suggesting that the sulfide fraction consisted mainly of cysteine. In the dark, even less organic sulfide was produced in the absence of *O*-acetylserine than in its presence. The light-dependent formation of organic sulfides was completely suppressed by 50 μM DCMU (data not shown).

Sulfite reduction in the light is accompanied by O_2 evolution, whereas sulfite oxidation consumes O_2. It has recently been reported that intact spinach chloroplasts exhibit net sulfite-dependent O_2 evolution in the light when radical scavengers such as SOD and catalase or ascorbate are present (9). On the other hand, light-dependent O_2 uptake was observed when radical scavengers were not added to the chloroplast suspensions. Although added catalase and SOD do not enter the chloroplasts, SOD is present and ascorbate levels are high in chloroplasts *in situ.*

Relatively fast light-dependent sulfite reduction (as documented in Fig. 3), and partial suppression of sulfite oxidation by SOD, suggest that *in vivo* sulfite reduction could be the preferred pathway of SO_2 detoxification. However, such a conclusion cannot be generalized. It does not agree with the observation that often the dominant product of SO_2 metabolization after fumigation of leaves with SO_2 is sulfate (7, 8, 14). The capacity of chloroplasts from different plants for sulfite reduction appears to be different. Moreover, reduction and introduction into organic compounds depends on the availability of *O*-acetylserine, of light and of sinks for organic sulfides. Although some accumulation of organic sulfides has been observed in plants after SO_2 fumigation (7, 8), cysteine does not belong to the group of amino acids that can be stored in appreciable quantities (23). Its main fate is incorporation into newly synthesized proteins and glutathione. Effective reductive SO_2 detoxification therefore depends on active pro-

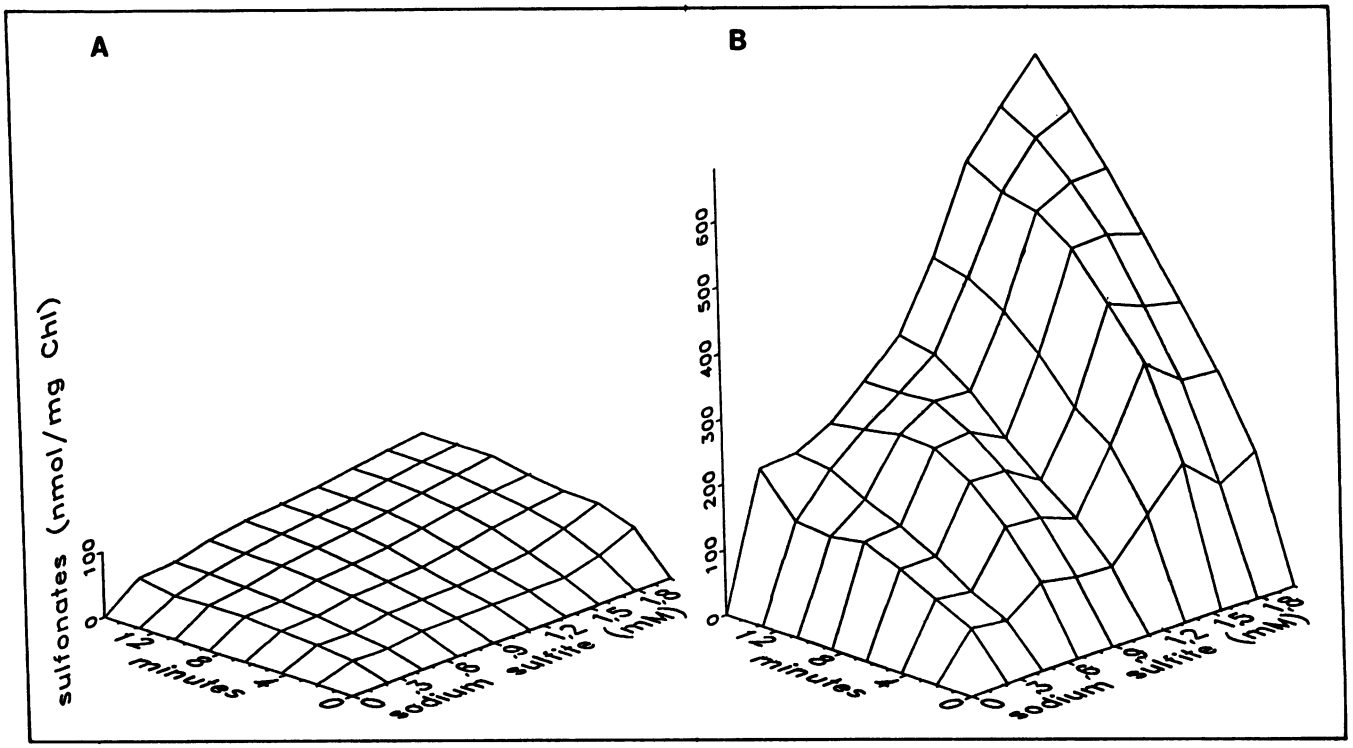

Figure 5. Concentration of sulfite addition compounds in suspensions of isolated spinach chloroplasts when 10 mM OAS was present in the light (A) and when it was absent (B).

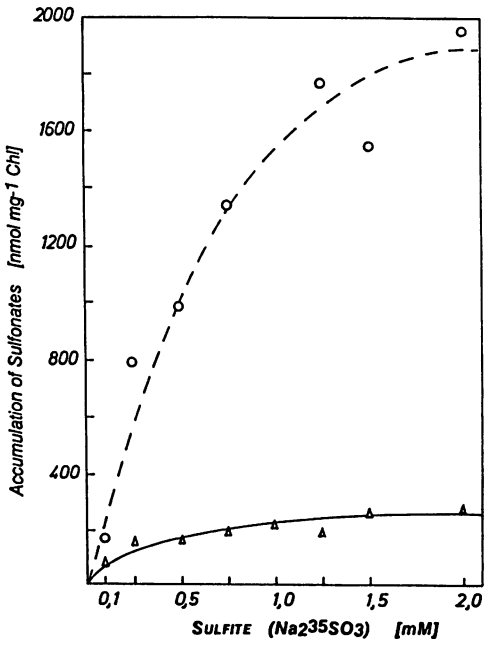

Figure 6. Saturation levels of sulfite addition compounds in the steady state between formation and breakdown after the addition of sulfite. △——△, In the presence of OAS in the light; O- - -O, without OAS in the light.

tein and glutathione synthesis, *i.e.* on growth. As a matter of fact, it has long been known that old trees are more sensitive to SO_2 than young trees that grow fast. Also, in areas where Norway spruce is slowly dying from overexposure to SO_2, grasses such as *Molinia coerulea* and *Deschampsia caespitosa* are luxuriant. Grasses contain much more protein in the dry mass than needles. Moreover, their growth period is extended compared to that of the conifers.

Accumulation of Sulfite Addition Compounds

Obviously, reduction can solve the problem of sulfite toxicity only when it is fast enough to compete successfully with reactions of sulfite, which are potentially damaging. Sulfite is a reactive solute. It is capable of forming addition compounds with, for instance, cellular aldehydes. It can also attack bisulfide bridges in proteins (19). α-Hydroxysulfonates are known to have inhibitory effects on metabolic reactions (27).

Sulfite can be liberated from its addition components by proper reductants. We have used dithiothreitol to measure the accumulation of undefined addition compounds in chloroplast suspensions. Figure 5 shows the accumulation of sulfite addition compounds in the light when OAS was either added to the chloroplast suspensions (Fig. 5A), or when it was absent (Fig. 5B). From a comparison with the sulfide data of Figure 3, it is readily apparent that addition compounds did not accumulate when the presence of *O*-acetylserine opened the pathway for cysteine synthesis in the light. Under these conditions, reduction competed successfully with addition

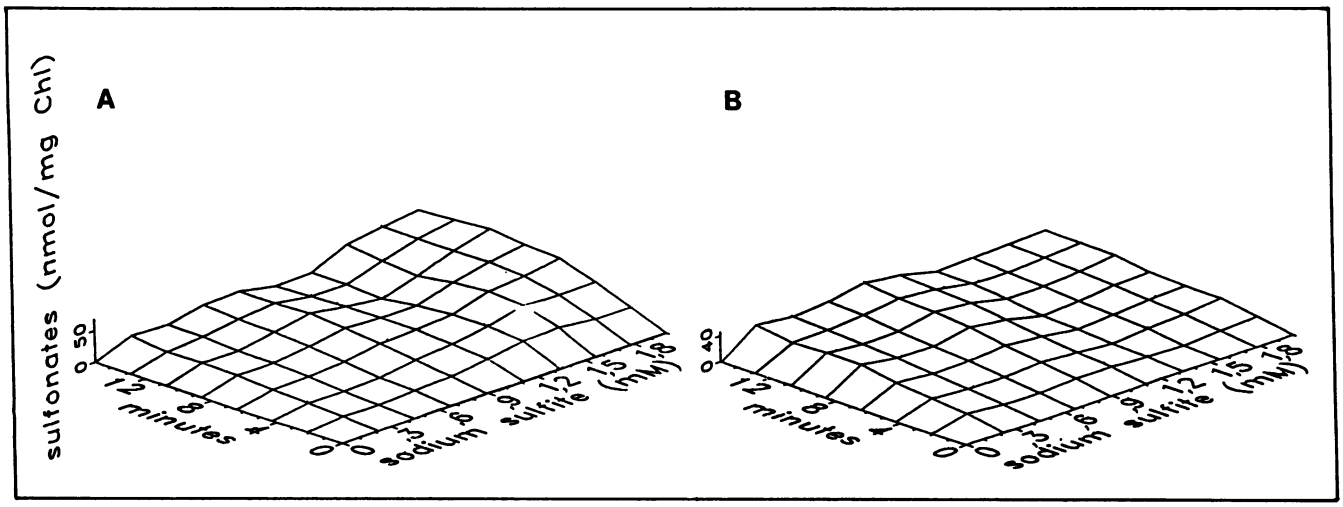

Figure 7. Concentration of sulfite addition compounds in isolated spinach chloroplasts when 10 mM OAS was present in the dark (A) and when it was absent (B).

reactions for available sulfite. However, when in the absence of OAS little synthesis of organic sulfides could take place in the light, addition compounds accumulated. A double reciprocal plot of concentration of the addition compounds *versus* time yielded straight lines for different sulfite concentrations (data not shown), indicating that concentrations of the addition compounds approached a saturation level. Saturation may be explained as a steady state situation in which synthesis and degradation (dissociation) balance each other. In contrast to the addition compounds, cysteine accumulated linearly with time in the chloroplast suspensions during its formation from sulfite and OAS (9).

When maximum concentrations of the addition compounds, as obtained by double reciprocal plots from Figure 5, were plotted against sulfite concentration, the results shown in Figure 6 were obtained. It can be seen that maximum concentrations of the addition compounds in the chloroplast suspensions were below 0.3 μmol·(mg Chl)$^{-1}$ when OAS was present in the light, but close to 2 μmol (mg Chl)$^{-1}$, when it was absent. This high concentration precludes binding of sulfite to chloroplast components. Although sulfite is known to be capable of reacting with hydrogen sulfide to form thiosulfate, this solute cannot be a significant component of the addition compounds, because thiosulfate is unstable in hydrochloric acid and would have been decomposed during acidification of the chloroplast extracts. At present, the molecular nature of the addition compounds is unknown.

Still, some conclusions can be drawn. Formation of the addition compounds is light-dependent (Fig. 5 *versus* Fig. 7). In the light, accumulation was largely, but not completely, inhibited by the electron transport inhibitor DCMU (50 μM, data not shown). Apparently, formation of the compounds involves either reductive reactions or depends on the production of radicals.

A very important conclusion from the accumulation of sulfite addition compounds in the absence, but not in the presence of *O*-acetylserine is that hydrogen sulfide, which has not been measured directly, apparently did not accumulate in the chloroplast suspensions in the light when *O*-acetylserine was absent. If it had been synthesized in the absence of OAS, the competitive relationship between synthesis of addition compounds and of organic sulfides would be very difficult to explain.

Formation of organic sulfides from sulfite can solve the problem of SO_2 toxicity. *In vivo* production of organic sulfides appears to depend on demand by sinks, *i.e.* on growth. If reduction is not possible, oxidation to sulfate is the main step of SO_2 metabolization. Oxidation occurs already in the dark and is accelerated in the light. The light-dependent reaction creates toxic radicals. Oxidation can only be considered a detoxification reaction if radicals are rapidly scavenged, and if it does not overload the cells with sulfate and protons (20, 21). High chloroplast concentrations of sulfate are known to inhibit photosynthesis (4). As a matter of fact, sulfate is sequestered inside the cells. In an ATP-dependent reaction, it is transported into the vacuole (16). In needles of spruce growing in the Erzgebirge near the Czechoslovak border, where atmospheric SO_2 concentrations are high, concentrations of sulfate in the cell sap were determined by us to be occasionally higher than 100 mM. Inasmuch as it is likely that the major part of this sulfate originates from SO_2, up to 200 μeq H^+ mL^{-1} cellular solution were produced during hydration and oxidation of the pollutant. Because cellular metabolism is pH-sensitive and the buffering capacity of leaf cells is insufficient to cope with the formation of such massive amounts of protons (24), mobilization of base is a necessity for cellular survival. The fate of an organism is decided by whether or not it is capable of neutralizing the sulfuric acid formed during hydration and oxidation of SO_2 even if detoxification of sulfite and bisulfite has been fast enough to prevent damage by these reactive anions.

ACKNOWLEDGMENT

We mourn the loss of a gifted young colleague and friend. Andreas Dittrich died on June 10, 1991, in a traffic accident.

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